EFFECT OF SALTS ON TOBACCO MOSAIC VIRUS RECONSTITUTION

Geneviève LEBEURIER, Michel LONCHAMPT and Léon HIRTH
Laboratoire des Virus des Plantes, Institut de Botanique de l'Université Louis Pasteur, 28, rue Goethe, 67083 Strasbourg Cédex, France

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1. Introduction

In vitro tobacco mosaic virus (TMV) reconstitutions performed by different groups [1–7] are generally made in sodium pyrophosphate buffer (NaPP) according to the conditions described by Fraenkel-Conrat and Singer [2]. It was later demonstrated [7–8] that in these conditions (0.1 M NaPP corresponding to 0.67 ionic strength (IS), and pH 7.25) dissociated TMV protein (4 S) aggregates very rapidly to form double discs which participate in both initiation and elongation [4–9]. In this paper we demonstrate that salts other than NaPP or Na phosphate are able to promote TMV reconstitution at the same pH but at lower ionic strength. In these defined conditions we obtain a good yield and the quality of the reconstituted rods is the same as that of the NaPP reconstituted material.

2. Material and methods

2.1. *Source of TMV proteins*

The technique, the purity criteria used and the coefficient employed to evaluate the protein concentration have been published elsewhere [9].

2.2. *Polymerization of the protein in NaCl or KCl buffer*

The required IS was obtained by adding various quantities of 1.0 IS NaCl or KCl buffer adjusted to pH 7.50 with 0.01 M Tris–HCl. After addition of protein the pH was 7.10 (±0.15). Tris–HCl at the same molarity had no effect on the polymerization of the protein.

The mixture was incubated for 10 min at 24°C, centrifuged at low speed, and the supernatant was analyzed by analytical ultracentrifugation. The S_{20w} values were calculated taking into account the viscosity and the density of the buffer but were not extrapolated to infinite dilution.

2.3. *The reaction mixture*

The reaction mixture for rod assembly was: 1 ml of TMV–RNA (100 μg/ml) in NaCl or KCl buffer at the IS chosen was added to 1.2 ml (1.8 mg/ml) of preincubated protein in the same buffer. The mixture was maintained at 24°C for 2 hr. The reconstitution was studied at 0.05, 0.1, 0.2 and 0.5 IS at pH 7.25. As control the same experiments were performed in NaPP.

2.4. *RNAase treatment*

The reconstituted material was ultracentrifuged. After the first ultracentrifugation [3] the pellets were resuspended in double distilled water and treated with RNAase for 10 min at 37°C (0.14 μg RNAase/A_{260}). After RNAase treatment, the reconstituted material was ultracentrifuged for 2 hr at 105 000 *g* as was the untreated material. The pellets were suspended in 0.01 M phosphate buffer pH 7.0.

2.5. *Reconstitution yield*

The reconstitution yield was estimated by the method of Stussi et al. [3] or in some cases by following the increase in light scattering (A_{310}) which is approx. proportional to the rod length and concentration of the particles over the range of the reaction [10]. The absorbance was recorded with a Beckman Acta III recording spectrophotometer.

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2.6. *Electron microscopy*

This was done according to the previously described method [3] and the histograms established from micrographs. The length of the particles was measured with an error of the order of 1 mm on photographs at 120 000 × (1 mm = 83 Å). From the histograms percentages of particle weights in various length categories were calculated.

2.7. *Bioassay*

Native and reconstituted TMV in 0.01 M phosphate buffer pH 7.0 were diluted in the same buffer at chosen concentrations to obtain between 10 and 100 local lesions per half leaf. Before inoculation, 25 μl of aqueous bentonite (10 mg/ml) were added to 2 ml of each solution. The TMV suspensions were assayed on the local lesion host (*N. tabacum* var *Xanthi necroticum*) using Latin square inoculation of at least 8 half leaves for each suspension tested.

3. Results

3.1. *Polymerization of TMV protein in NaCl or KCl at pH 7.25 at different ionic strengths*

After 10 min of incubation at pH 7.25, 0.025 and 0.05 IS NaCl or KCl, the protein clustered into heterogeneous component (4–8 S). Between 0.1 and 0.3 IS the protein polymerized quickly to form a large amount of 25 S aggregates with little protein remaining in the 4–8 S aggregates (fig. 1a, b). At 0.5 IS and above a 34 S component appeared (fig. 1c). Even at very low IS (0.05) and particularly in KCl a small peak of 20 S was obtained after 2 hr of incubation (fig. 1d). Consequently NaCl or KCl buffer were apparently very efficient in the formation of the protein structure necessary for both initiation and elongation [9].

3.2. *Reconstitution of protein plus RNA in NaCl or KCl buffer at pH 7.25*

The reconstitution yield in NaCl or KCl buffer was high at low ionic strength (0.1 and 0.2 IS), but was less at 0.5 IS (table 1). In contrast, reconstitution in NaPP was poor in 0.1 and 0.2 IS, but greatly increased at 0.5 IS. Even at 0.05 IS in NaCl and especially in KCl, the yield was high provided that the 4 S protein was incubated previously for 2 hr in the buffer at the same IS.

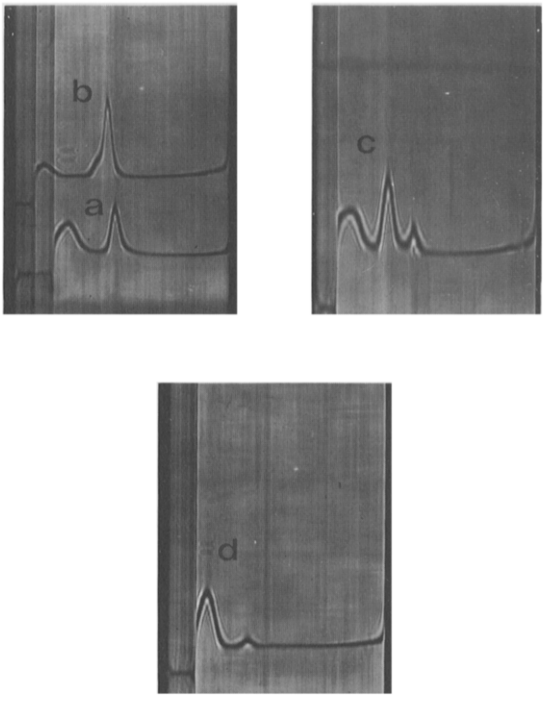

Fig. 1. Sedimentation patterns of TMV protein in NaCl or KCl buffer adjusted to pH 7.25 with 0.01 M Tris–HCl. All photographs were taken 16 min after the rotor reached a speed of 47 600 rpm. Sedimentation was from left to right; a–b–c = Protein after incubation for 10 min in NaCl buffer at 0.1, 0.2 or 0.5 IS respectively A_{20w}. a = (4–8) and 20; b = (4–8) and 25; c = (4–8) – 25 and 34; d = Protein after incubation for 2 hr in KCl buffer 0.05 IS. S_{20w} = 8 and 20. The protein concentration was 4 mg/ml for each sample.

Table 1

Reconstitution yield as a function of the ionic strength at pH 7.25 in various buffers.

Reconsitution buffer	% Of sedimentable (i.e. coated) RNA at various IS			
	0.05	0.1	0.2	0.5
NaPP	–	3	4	59
NaCl	19	53	55	30
KCl	36	45	50	15

The reconstitution period was 2 hr at 24°C. The protein used was previously incubated for 10 min in the same buffer except for the IS 0.05 where the incubation was 2 hr.

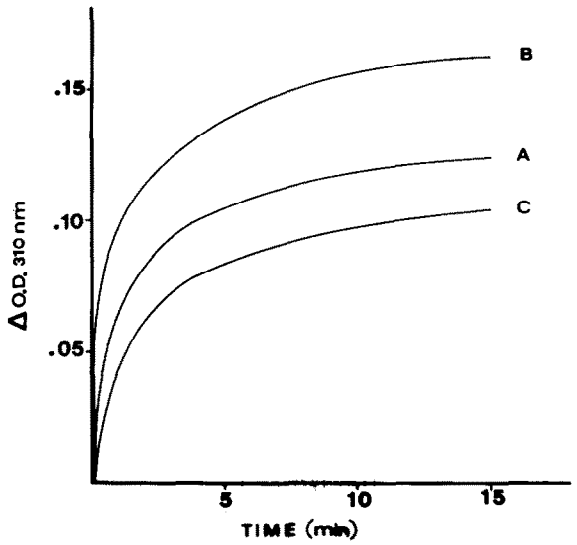

Fig. 2. Kinetics of reconstitution followed by light scattering at 310 nm. Protein was incubated for 10 min in various buffers at pH 7.25 and centrifuged at low speed. For each sample 50 μg RNA (1 mg/ml) were added to 1 mg preincubated protein (1 mg/ml): A = reconstitution in NaPP buffer 0.5 IS; B = reconstitution in KCl buffer 0.2 IS; C = reconstitution in NaCl buffer 0.2 IS.

Table 2
RNAase sensitivity of the reconstituted material in various buffers at pH 7.25.

IS of reconstitution buffer	Yield of sedimentable material in % of RNA coated	
	−RNAase	[a] +RNAase
0.5 NaCl	40	25
0.2 NaCl	55	52
0.2 KCl	35	35

[a] After RNAase treatment the untreated and treated samples, were ultracentrifuged for 2 hr at 105 000 g. The pellets were suspended in phosphate buffer pH 7.0, 0.01 M.

The reconstitution kinetics were studied by turbidometry at 310 nm. The results obtained are shown in fig. 2. The reconstitution was good in the three buffers: (0.5 IS NaPP and 0.2 IS NaCl or KCl) and especially in KCl.

3.3. *RNAase sensitivity*

For a given IS, the RNAase stability of the reconstituted material depended on the nature of the salt. For example the material reconstituted in NaPP buffer 0.5 IS was RNAase stable, although it was

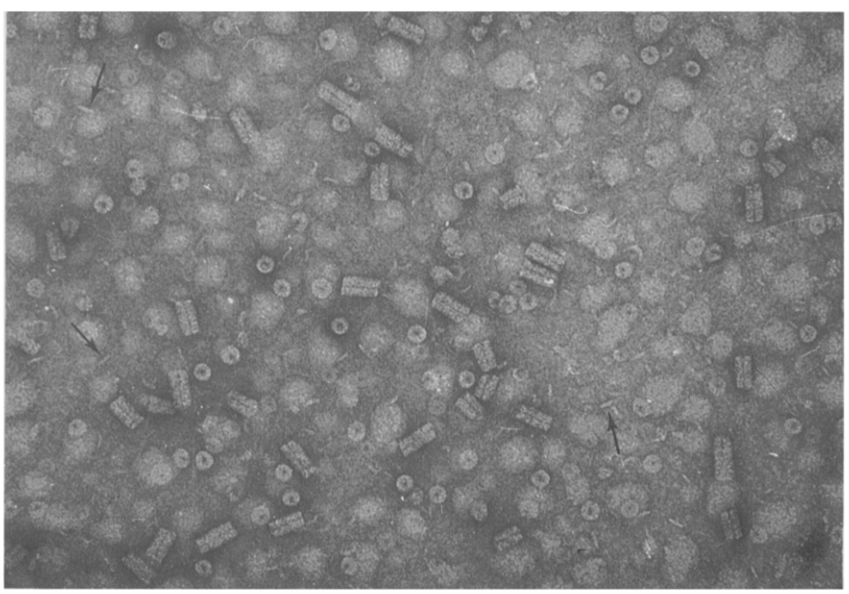

Fig. 3. Electron micrograph of reconstituted material in NaCl buffer 0.5 IS after RNAase treatment. The RNAase was added to the reconstitution mixture after 2 hr of incubation. Negative staining with 1% uranyl formate (×120 000). Arrows indicate some structures which are called 'fish' by Durham and Finch [11].

Table 3
Specific infectivity of reconstituted material in various buffers before and after RNAase treatment.

Reconstitution buffer	Concentration of each inoculum in μg/ml to obtain 20 lesions per half-leaf	
	−RNAase	+RNAase
Original TMV	0.005	0.005
NaPP 0.5 IS	0.015	0.015
NaCl 0.5 IS	0.03	0.2
NaCl 0.2 IS	0.015	0.015
KCl 0.2 IS	0.02	0.02

RNAase sensitive when reconstituted in 0.1 IS [9] or 1.0 IS (unpublished results). Similarly, material reconstituted in NaCl or KCl at 0.2 IS was RNAase stable, but RNAase sensitive at 0.05 or 0.5 IS (table 2).

The results obtained by electron microscopy were in accord with those shown in table 2. In NaCl or KCl reconstitutions at 0.2 IS the proportion (by weight) of 3000 Å rods remained unchanged after RNAase treatment. In 0.5 IS the percentage of 3000 Å rods was of the same order, but was decreased by RNAase treatment by about half.

After NaPP 1.0 IS or NaCl 0.5 IS reconstitution and when RNAase treatment was performed directly in rhe reconstitution medium, a large amount of double discs and 'fishes' [11] were observed (fig. 3) in the supernatant obtained after ultracentrifugation.

3.4. *Infectivity of the reconstituted material*

Table 3 summarizes the infectivities of the different reconstitution samples. At 0.5 IS the virus reconstituted in NaCl buffer was six times less infectious than the original virus and half as infectious as the material reconstituted in NaPP at the same IS. The specific infectivity of the control (NaPP buffer 0.5 IS) was not affected by RNAase treatment, but the material reconstituted in NaCl buffer, at the same IS suffered a drastic decrease (7 times) after RNAase treatment. A similar decrease of the specific infectivity was observed with material reconstituted in NaPP buffer at 1.0 IS.

The material reconstituted in 0.2 IS NaCl or KCl was two or four times less infectious than the original virus solution depending on different experiments. However, these particles had approximately the same specific infectivity before and after RNAase treatment. In fact the reconstituted rods in 0.2 IS NaCl or KCl were as infectious as those reconstituted in NaPP 0.5 IS. The material reconstituted in 0.05 IS in NaCl or KCl was much less infectious than the original virus solution and its specific infectivity decreased much more after RNAase treatment.

4. Discussion

Till now the TMV reconstitution has been carried out in phosphate or NaPP buffers as these were considered the most suitable. We demonstrate that other ions are able to promote a rapid aggregation of the 4 S protein into 20 or 25 S aggregates corresponding to double discs or at least to structures very efficient in TMV reconstitution. Optimal efficiency of reconstitution occurs, in KCl or NaCl, at a lower IS than in NaPP especially if the optimal conditions are related both to the yield and the quality of the reconstituted material. In fact we have observed that the reconstitution yield and the properties of the material obtained are similar in NaPP 0.5 IS and in KCl or NaCl 0.2 IS. This point is important if we remember that the average value of the IS of tobacco cell as measured by osmotic equilibrium is approximately 0.2 [12] and that the KCl is present in large amount in plant cells. It seems that reconstitution in NaCl and especially in KCl would be very near the physiological conditions. It is interesting to observe that the optimal IS for protein aggregation and TMV reconstitution is lower in KCl or NaCl than in phosphates. This seems to indicate that if ionic interactions play an important role in these polymerizations, the contribution of other forces cannot be neglected. This point will be discussed elsewhere.

Acknowledgements

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